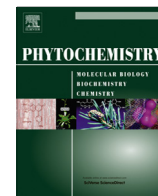


Contents lists available at [SciVerse ScienceDirect](http://SciVerse.ScienceDirect.com)

## Phytochemistry

journal homepage: [www.elsevier.com/locate/phytochem](http://www.elsevier.com/locate/phytochem)

# Bioactivity against *Bursaphelenchus xylophilus*: Nematotoxics from essential oils, essential oils fractions and decoction waters

Jorge M.S. Faria<sup>a</sup>, Pedro Barbosa<sup>b</sup>, Richard N. Bennett<sup>c</sup>, Manuel Mota<sup>b,d</sup>, A. Cristina Figueiredo<sup>a,\*</sup>

<sup>a</sup> Universidade de Lisboa, Faculdade de Ciências de Lisboa, DBV, IBB, Centro de Biotecnologia Vegetal, C2, Campo Grande, 1749-016 Lisboa, Portugal

<sup>b</sup> NemaLab, ICAAM – Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Núcleo da Mitra, Ap. 94, 7002-554 Évora, Portugal

<sup>c</sup> Universidade de Trás-os-Montes e Alto Douro, Quinta dos Prados – Apartado 1013, 5000-911 Vila Real, Portugal

<sup>d</sup> INIAV/Unidade Estratégica de Investigação e Serviços de Sistemas Agrários e Florestais e Sanidade Vegetal, Av. da República, Quinta do Marquês, 2784-159 Oeiras, Portugal

## ARTICLE INFO

## Article history:

Received 22 February 2013

Received in revised form 29 May 2013

Available online 2 July 2013

## Keywords:

*Bursaphelenchus xylophilus*

*Pinus pinaster*

Essential oils

Hydrocarbon molecules

Oxygen-containing molecules

Decoction waters

Nematotoxic

2-Undecanone

Carvacrol

$\gamma$ -Terpinene

## ABSTRACT

The Portuguese pine forest has become dangerously threatened by pine wilt disease (PWD), caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. Synthetic chemicals are the most common pesticides used against phytoparasitic nematodes but its use has negative ecological impacts. Phytochemicals may prove to be environmentally friendly alternatives. Essential oils (EOs) and decoction waters, isolated from 84 plant samples, were tested against *B. xylophilus*, in direct contact assays. Some successful EOs were fractionated and the fractions containing hydrocarbons or oxygen-containing molecules tested separately. Twenty EOs showed corrected mortalities  $\geq 96\%$  at 2  $\mu\text{L/mL}$ . These were further tested at lower concentrations. *Ruta graveolens*, *Satureja montana* and *Thymbra capitata* EOs showed lethal concentrations ( $\text{LC}_{100}$ )  $< 0.4 \mu\text{L/mL}$ . Oxygen-containing molecules fractions showing corrected mortality  $\geq 96\%$  did not always show  $\text{LC}_{100}$  values similar to the corresponding EOs, suggesting additive and/or synergistic relationships among fractions. Nine decoction waters (remaining hydrodistillation waters) revealed 100% mortality at a minimum concentration of 12.5  $\mu\text{L/mL}$ . *R. graveolens*, *S. montana* and *T. capitata* EOs are potential environmentally friendly alternatives for *B. xylophilus* control given their high nematotoxic properties. Nematotoxic activity of an EO should be taken in its entirety, as its different components may contribute, in distinct ways, to the overall EO activity.

© 2013 Elsevier Ltd. All rights reserved.

## 1. Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* L. is a highly pathogenic plant parasite that infects, mainly, *Pinus* species, and causing pine wilt disease (PWD) (Mota and Vieira, 2008). In 1999, Portugal became its entry point to the European pine forests (Mota et al., 1999). Since then, this phytoparasite has been progressing through the country, having been found in Madeira Island, in 2010 (Fonseca et al., 2012), and in Spain, in 2011 (Abelleira et al., 2011). It has been classified as an A2 type quarantine pest by the European Plant Protection Organization (EPPO, 2012). In Portugal, *Pinus pinaster* Aiton, maritime pine, is the susceptible species.

Phytoparasitic nematode control is very complex, generally relying upon synthetic chemicals, as broad-spectrum nematicides which are extremely damaging (Chitwood, 2003). The PWN is commonly controlled by controlling the insect vector through aerial application of synthetic insecticides, by fumigation of infected trees, pine tree-free strips, use of vector natural enemies, or by

controlling the nematode through trunk injection of anti-nematicidal compounds such as abamectins (Lee et al., 2003; Takai et al., 2003; Mota and Vieira, 2008). Nevertheless, the use of synthetic pesticides is associated with environment pollution and undesirable influences on human health or against non-target organisms (Zhao, 2008). The use of plant derived natural products for pest control is not recent, but has gained ground in modern pest management due to growing ecological concerns about the use of synthetic pesticides, and their consequent phasing out in the market and banning. Essential oils (EOs) are complex mixtures of volatiles, mainly products of plant secondary metabolism. Commonly, they are comprised of terpenes, mono- and sesquiterpenes, and phenolic compounds, such as phenylpropanoids, although other groups of compounds can also occur in relevant amounts. They are generally biodegradable, have low toxicity to mammals and do not accumulate in the environment (Figueiredo et al., 2008). The biological activities of EOs can frequently exceed the sum of their single constituent's activities, due to synergy. As complex mixtures, EOs may display several biological activities which makes them desirable biopesticides, being able to control not just the targeted pest but also opportunistic species and resistant strains. This is of particular interest in phytoparasitic nematode

\* Corresponding author. Tel.: +351 217500257.

E-mail addresses: [acf@fc.ul.pt](mailto:acf@fc.ul.pt), [acsf@fc.ul.pt](mailto:acsf@fc.ul.pt) (A.C. Figueiredo).

control since complex disease symptoms are also commonly associated with accompanying pathogenic microbiota (Back et al., 2002; Vicente et al., 2011). Several EOs, such as those of *Cinnamomum zeylanicum* (Kong et al., 2006); *Boswellia carterii*, *Paeonia moutan*, *Perilla frutescens*, *Schizonepeta tenuifolia* (Choi et al., 2007a); *Thymus vulgaris* (Kong et al., 2007); *Litsea cubeba*, *Pimenta dioica*, *Trachyspermum ammi* (Park et al., 2007); *Coriandrum sativum* and *Liquidambar orientalis* (Kim et al., 2008) have revealed strong activities against *B. xylophilus*.

Containing this pest is of the utmost importance for the European pine forest safeguard. With the purpose of finding alternative means of controlling this phytoparasite, without further destabilizing the forest ecosystem, the present study was aimed at screening several plant taxa, some of which from the Portuguese flora, for natural phytochemicals with PWN nematotoxic properties. In view of this, (a) essential oils and decoction waters (remaining hydrodistillation water), isolated from 84 samples, were evaluated through direct contact assays, and (b) fractions containing hydrocarbons or oxygen-containing molecules from the most successful essential oils were further assessed, separately, against the PWN.

## 2. Results and discussion

### 2.1. Composition of essential oils and fractions containing hydrocarbons or oxygen-containing molecules

The essential oils isolated from 59 plant species (13 families), revealed yields that ranged from <0.05% to 9% (v/w). The highest yields were obtained from *Syzygium aromaticum* (9%), *Eucalyptus radiata* (6%), *E. dives* (3%) and *Cymbopogon citratus* (3%) (Table 1).

All 84 EOs isolated were fully chemically characterized (detailed relative amounts of all the identified components are listed in the Supplementary Table, ST), although Table 1 reports only their main components ( $\geq 10\%$ ). For some species, duly identified in Table 1, the EO composition was previously reported (Barbosa et al., 2010, 2012; Faria et al., 2011). Some species can show several EO chemotypes, such as *Thymus caespitius* carvacrol, thymol or  $\alpha$ -terpineol-rich chemotypes (Table 1), which may provide different biological properties. These chemotypes were separately assessed for their nematotoxic activity.

Some EOs were further chosen for fractionation, aiming to assess the independent input of the EO fractions containing hydrocarbons or oxygen-containing molecules against PWN. The main components ( $\geq 10\%$ ) in each separate EO fraction are featured in Table 2 (detailed relative amounts are listed in the Supplementary Table, ST).

The high chemical diversity of the analyzed EOs, and of the corresponding fractions containing hydrocarbons or oxygen-containing molecules, was supported by the agglomerative cluster analysis based on their full chemical composition (Fig. 1). Despite this chemical diversity, the analyzed EOs were predominantly terpene-rich, although other chemical groups also achieved important percentages, such as in *Ruta graveolens* EO, dominated by the methyl nonyl ketone, 2-undecanone, or in *S. aromaticum* and in *Foeniculum vulgare* EOs, rich in the phenylpropanoids eugenol and *trans*-anethole, respectively (Table 1).

Cluster analysis showed two main uncorrelated clusters ( $S_{\text{corr}} < 0.2$ ) (Fig. 1). Cluster I with only five out of the 94 samples analyzed, included EOs characterized by high percentages of specific compounds, usually not present in such high amounts in the other EOs. This was the case of 2-undecanone (91–94%) in *R. graveolens*, eugenol (93%) in *S. aromaticum* and 4 $\alpha$ , 7 $\alpha$ , 7 $\alpha$ -nepetalactone (89%) in *Nepeta cataria* EOs.

Cluster II grouped the remaining EOs, and related fractions containing hydrocarbons or oxygen-containing molecules, represent-

ing 95% of the samples analyzed. Having been sub-divided into several sub-clusters, some of which also highly uncorrelated ( $S_{\text{corr}} < 0.2$ ), the essential oils from this cluster were predominately terpene-rich.

### 2.2. PWN mortality and LC<sub>100</sub> assessment

#### 2.2.1. Essential oils

Essential oils were tested for activity against *B. xylophilus* through direct contact bioassays. Assays, performed with ultrapure water, showed an average mortality of  $8 \pm 4\%$ , considered to be natural mortality. The mortality due to methanol, used as the EO solvent, was  $10 \pm 6\%$ , which can be considered negligible, when compared to natural mortality.

At the highest concentration, 2  $\mu\text{L/mL}$ , some of the ineffective EOs or EO fractions assessed, with corrected mortalities <40% showed dominant proportions of e.g. the monoterpenes limonene,  $\alpha$ -pinene, 1,8-cineole, camphor, terpinolene, or sabinene (Table 1, Table 2 and Fig. 1).

The most active EOs, or EO fractions, showing corrected mortalities  $\geq 96\%$  at 2  $\mu\text{L/mL}$ , occurred both in cluster I and in sub-clusters IIa, IIc, IIe, and IIq from cluster II (Table 1, Table 2 and Fig. 1). Within cluster I, *R. graveolens* (91–94% 2-undecanone) and *S. aromaticum* (93% eugenol) EOs were highly effective (100% corrected mortality).

Sub-cluster IIa included EOs, or fractions with oxygen-containing molecules, with  $\geq 93\%$  corrected mortality, which were chemically characterized by dominant contents of carvacrol (35–96%), *p*-cymene (traces–20%),  $\gamma$ -terpinene (traces–18%) and thymol (not detected–15%). Only three out of the seven samples from sub-cluster IIc showed corrected mortalities  $\geq 96\%$ . These differed from the remaining members of the same cluster by showing high amounts of thymol (12–23%) and carvacrol (6–15%). Sub-cluster IIe integrated EOs rich in thymol (42–50%), *p*-cymene (14–20%), thymol acetate (traces–15%) and  $\gamma$ -terpinene (6–12%), which showed corrected mortalities  $\geq 99\%$ . *Th. caespitius* chemotypes rich in carvacrol and/or thymol showed high nematotoxic activities while  $\alpha$ -terpineol-rich chemotypes showed corrected mortalities  $\leq 60\%$ . The occurrence of chemotypes must be taken into account when choosing a nematotoxic EO bearing-species, since EO particular chemotype proved to be determinant in nematotoxic activity.

High PWN mortality (100%) was also observed with *C. citratus* EOs, and the related oxygen-containing molecules fraction. These EOs grouped in sub-cluster IIq and were dominated by geranial (23–45%),  $\beta$ -myrcene (traces–38%), neral (20–36%), and geraniol (1–18%).

Three other EOs showed corrected mortalities  $\geq 96\%$ , although this toxicity was not shown by the EOs of other members of the same sub-clusters. *Mentha cervina* (96% corrected mortality) characterized by high contents of pulegone (80%), *E. citriodora* (97% corrected mortality) citronellal (36%), isopulegol (13%), citronellol (12%) and 1,8-cineole (11%) rich, and *M. arvensis* EOs (100% corrected mortality), dominated by piperitenone oxide (56%).

Despite the chemical diversity of the assessed EOs, it is noteworthy that sub-clusters IIa, IIc and IIe gathered EOs that had in common the presence of carvacrol, thymol, *p*-cymene and/or  $\gamma$ -terpinene. Separately or combined, these compounds can be partially responsible for each EOs nematotoxic properties.

EOs that attained nematotoxic activity  $\geq 96\%$ , namely those of *C. citratus* 1, 2, and 3, *E. citriodora*, *M. arvensis*, *M. cervina* 1, *Origanum vulgare* subsp. *virens*, *Origanum vulgare* 2, *R. graveolens* 1, 2 and 3, *Satureja montana* 1 and 2, *S. aromaticum*, *T. capitata*, *Th. caespitius* 2, 5 and 7 (carvacrol and/or thymol-rich), *Th. vulgaris* and *Th. zygis* were further tested at lower concentrations (Table 1).

**Table 1**

Plant species scientific names, arranged in alphabetic order of the corresponding plant family, sampling year, plant part used for hydrodistillation, plant source, essential oil yield and EO main components ( $\geq 10\%$ ) of each of the 84 EOs analyzed.

Family/species	Code	Sampling date	Plant part	Collection place/source	Yield (% v/w)	Main components (%)
<b>Apiaceae/Umbelliferae</b>						
<i>Angelica lignescens</i> Reduron et Danton <sup>a,b</sup>	Al	2008	FV	Flores, Azores	0.08	limonene 65, $\beta$ -phellandrene 13, $\beta$ -myrcene 12
<i>Apium graveolens</i> L. <sup>b</sup>	Ag	2010	FV	Lisbon	0.05	limonene 92
<i>Chaerophyllum azoricum</i> Trel. <sup>a,b</sup>	Ca	2008	FV	Flores, Azores	0.25	terpinolene 30, myristicin 26, acorenone B 17
<i>Foeniculum vulgare</i> Mill. <sup>a,b</sup>	Fv	2008	FF	Graciosa, Azores	0.33	<i>trans</i> -Anethole 73, $\alpha$ -pinene 13
<i>Petroselinum crispum</i> (Mill.) Nym. <sup>b</sup>	Pc	2009	FV	Lisbon	0.09	1,3,8- <i>p</i> -Menthatriene 50, $\beta$ -myrcene 13, apiole 11
<b>Asteraceae/Compositae</b>						
<i>Achillea millefolium</i> L.	Am	2010	DF	Herbal shop	0.85	$\beta$ -Thujone 33, <i>trans</i> -chrisantenyl acetate <sup>e</sup> 19
<i>Inula viscosa</i> (L.) Aiton <sup>b</sup>	Iv	2009	FV	Lisbon	<0.05	1,8-Cineole 30
<b>Cupressaceae</b>						
<i>Cryptomeria japonica</i> (Thunb. ex L.f.) D. Dona <sup>a,b</sup>	Cj	2008	Ffruit	Flores, Azores	0.41	Terpinen-4-ol 24, $\alpha$ -pinene 23, sabinene 17
<i>Juniperus brevifolia</i> (Seub.) Antoine 1 <sup>a,b</sup>	Jb1	2008	Ffruit	Flores, Azores	0.06	Limonene 63, $\alpha$ -pinene 18
<i>Juniperus brevifolia</i> (Seub.) Antoine 2 <sup>a</sup>	Jb2	2008	FV	Flores, Azores	0.45	Limonene 82, $\alpha$ -pinene 11
<b>Fabaceae/Leguminosae</b>						
<i>Genista tridentata</i> L.	Gt	2010	DV	Herbal shop	<0.05	<i>cis</i> -Theaspirane 27, <i>trans</i> -theaspirane 22
<b>Geraniaceae</b>						
<i>Pelargonium graveolens</i> L'Hér. <sup>a,b</sup>	Pg	2009	FV	Lisbon	0.19	Citronellol 34, guaia-6,9-diene 15, citronellyl formate <sup>e</sup> 14
<b>Lamiaceae/Labiatae</b>						
<i>Calamintha nepeta</i> (L.) Savi <sup>b</sup>	Cn	2009	FF	Castelo Branco	1.43	Isomenthone 52, isomenthol 19, 1,8-cineole 11
<i>Melissa officinalis</i> L. <sup>a,b</sup>	Mo	2009	FF	Herbal shop	0.04	Geranial 38, citronellol 32, geraniol 18
<i>Mentha arvensis</i> L.	Ma	2009	FV	Lisbon	0.06	Piperitenone oxide 56
<i>Mentha cervina</i> L. 1 <sup>b</sup>	Mc1	2009	DV	Castelo Branco	0.80	Pulegone 80
<i>Mentha cervina</i> L. 2 <sup>b</sup>	Mc2	2009	DF	Castelo Branco	1.10	Pulegone 86
<i>Mentha x piperita</i> L. 1 <sup>b</sup>	Mp1	2009	FV	Lisbon	0.11	Menthol 31, menthone 19
<i>Mentha x piperita</i> L. 2	Mp2	2009	FV	Lisbon	0.73	Menthone 56, pulegone 13
<i>Mentha pulegium</i> L.	Mpu	2008	DV	Lisbon	0.35	Pulegone 49, piperitenone 10
<i>Mentha spicata</i> L. 1 <sup>b</sup>	Ms1	2009	FV	Lisbon	0.07	Carvone 54
<i>Mentha spicata</i> L. 2 <sup>a</sup>	Ms2	2009	FV	Beja	0.25	Carvone 70
<i>Nepeta cataria</i> L. <sup>a</sup>	Nc	2009	FF	Herbal shop	0.18	4 $\alpha$ , 7 $\alpha$ , 7 $\alpha$ -Nepetalactone 89
<i>Origanum majorana</i> L.	Om	2010	FV	Coimbra	0.06	<i>cis</i> -Sabinene hydrate 33, terpinen-4-ol 13
<i>Origanum vulgare</i> L. 1	Ov1	2010	FV	Coimbra	<0.05	Carvacrol 14, <i>cis</i> -sabinene hydrate 14, $\gamma$ -terpinene 10
<i>Origanum vulgare</i> L. 2	Ov2	2010	DL	Herbal shop	1.00	$\alpha$ -Terpineol 16, thymol 15, $\gamma$ -terpinene 15, carvacrol 10
<i>Origanum vulgare</i> subsp. <i>virens</i> (Hoffmanns. and Link) Bonnier and Layens	Ovi	2010	DV	Herbal shop	0.83	$\alpha$ -Terpineol 40, linalool 16, thymol 12
<i>Rosmarinus officinalis</i> L. <sup>a,b</sup>	Ro	2009	DL	Herbal shop	1.95	$\beta$ -Myrcene 29, $\alpha$ -pinene 15
<i>Salvia officinalis</i> L. <sup>a,b</sup>	So	2009	FV	Lisbon	0.54	$\alpha$ -Thujone 29, 1,8-cineole 26, $\beta$ -thujone 10
<i>Satureja montana</i> L. 1 <sup>a,b</sup>	Sm1	2009	DV	Herbal shop	0.55	Carvacrol 40, <i>p</i> -cymene 20, thymol 15
<i>Satureja montana</i> L. 2 <sup>b</sup>	Sm2	2010	DF	Herbal shop	1.31	Carvacrol 64, $\gamma$ -terpinene 18
<i>Thymbra capitata</i> (L.) Cav. <sup>a,b</sup>	Tc	2010	FF	Algarve	1.40	Carvacrol 68, $\gamma$ -terpinene 11
<i>Thymus caespititius</i> Brot. 1 <sup>a,b</sup>	Thc1	2008	FF	Flores, Azores	0.06	Carvacrol 35, <i>p</i> -cymene 19
<i>Thymus caespititius</i> Brot. 2 <sup>a</sup>	Thc2	2008	FF	Corvo, Azores	0.22	Carvacrol 47, carvacrol acetate 12, T-cadinol 12
<i>Thymus caespititius</i> Brot. 3 <sup>a,b</sup>	Thc3	2008	FF	Gerês	0.35	$\alpha$ -Terpineol 36, <i>p</i> -cymene 13, $\gamma$ -terpinene 13
<i>Thymus caespititius</i> Brot. 4 <sup>a</sup>	Thc4	2008	FF	Graciosa, Azores	0.38	$\alpha$ -terpineol 62
<i>Thymus caespititius</i> Brot. 5 <sup>b</sup>	Thc5	2009	FF	Terceira, Azores	0.33	Thymol 42, thymol acetate 15, <i>p</i> -cymene 14
<i>Thymus caespititius</i> Brot. 6	Thc6	2004–2009	FF	Azores	<sup>c</sup>	Carvacrol 54, carvacrol acetate 10
<i>Thymus caespititius</i> Brot. 7	Thc7	2010	FF	Coimbra	0.48	Carvacrol 59, <i>p</i> -cymene 11
<i>Thymus camphoratus</i> Hoffmanns. and Link <sup>a,b</sup>	Thca	2008	FF	Algarve	0.21	Linalool 26, linalool acetate 18
<i>Thymus mastichina</i> (L.) L. <sup>b</sup>	Thm	2010	FV	Coimbra	1.17	1,8-Cineole 46, limonene 23
<i>Thymus villosus</i> subsp. <i>lusitanicus</i> (Boiss.) Coutinho <sup>a</sup>	Thvl	2008	FF	Leiria	1.25	Linalool 69
<i>Thymus vulgaris</i> L.	Thv	2010	FV	Coimbra	0.08	Thymol 48, <i>p</i> -cymene 20, $\gamma$ -terpinene 12
<i>Thymus zygis</i> L. <sup>b</sup>	Thz	2010	FV	Coimbra	0.30	Thymol 50, <i>p</i> -cymene 15
<i>Thymus zygis</i> subsp. <i>silvestris</i> (Hoffmanns. and Link) Coutinho 1 <sup>a,b</sup>	Thzs1	2008	FF	Leiria	0.23	$\alpha$ -Terpineol 32, $\gamma$ -terpinene 16, linalool 11
<i>Thymus zygis</i> subsp. <i>silvestris</i> (Hoffmanns. and Link) Coutinho 2 <sup>b</sup>	Thzs2	2008	FF	Santarém	0.94	$\alpha$ -Terpineol 60
<b>Lauraceae</b>						
<i>Cinnamomum camphora</i> (L.) Sieb. <sup>b</sup>	Cc	2009	FF	Coimbra	0.47	Camphor 49, $\alpha$ -pinene 10
<i>Laurus azorica</i> (Seub.) J. Franco <sup>a,b</sup>	La	2008	FV	Flores, Azores	0.25	$\alpha$ -Pinene 35, $\beta$ -pinene 16, <i>trans</i> - $\alpha$ -bisabolene 15
<i>Laurus nobilis</i> L. <sup>b</sup>	Ln	2009	DL	Herbal shop	0.95	1,8-Cineole 35, $\alpha$ -terpenyl acetate 13

Table 1 (continued)

Family/species	Code	Sampling date	Plant part	Collection place/ source	Yield (% v/w)	Main components (%)
<b>Myrtaceae</b>						
<i>Eucalyptus bosistoana</i> F. Muell. <sup>b,d</sup>	Eb	2009	FV	Santarém	1.80	1,8-Cineole 59, $\alpha$ -pinene 14
<i>Eucalyptus botryoides</i> Sm. <sup>b,d</sup>	Ebo	2009	FV	Santarém	1.20	$\alpha$ -Pinene 43, 1,8-cineole 35
<i>Eucalyptus camaldulensis</i> Dehnh. <sup>b,d</sup>	Ec	2009	FV	Santarém	1.30	1,8-Cineole 51, $\alpha$ -pinene 32
<i>Eucalyptus cinerea</i> F. Muell. <sup>d</sup>	Eci	2009	FF	Santarém	1.60	1,8-Cineole 67, $\alpha$ -terpinyl acetate 10
<i>Eucalyptus citriodora</i> Hook. <sup>b,d</sup>	Ect	2009	FV	Santarém	0.86	Citronellal 36, isopulegol 13, citronellol 12, 1,8-cineole 11
<i>Eucalyptus cordieri</i> Trabut <sup>d</sup>	Eco	2009	FV	Santarém	1.12	1,8-Cineole 72
<i>Eucalyptus dives</i> Schauer <sup>d</sup>	Ed	2009	FV	Santarém	3.30	Piperitone 40, $\alpha$ -phellandrene 19, <i>p</i> -cymene 19
<i>Eucalyptus ficifolia</i> F. Muell. <sup>b,d</sup>	Ef	2009	FV	Santarém	0.35	$\alpha$ -Pinene 44, limonene 41
<i>Eucalyptus globulus</i> Labill. <sup>d</sup>	Eg	2009	FV	Santarém	1.33	1,8-Cineole 70, $\alpha$ -pinene 16
<i>Eucalyptus pauciflora</i> Sieber ex Spreng <sup>d</sup>	Ep	2009	FF	Santarém	0.84	$\alpha$ -Pinene 82
<i>Eucalyptus polyanthemos</i> Schauer <sup>b,d</sup>	Epo	2009	FV	Santarém	0.55	1,8-Cineole 27
<i>Eucalyptus radiata</i> Sieber <sup>d</sup>	Er	2009	FV	Santarém	5.55	1,8-Cineole 48, <i>p</i> -cymene 13
<i>Eucalyptus saligna</i> Sm. <sup>b,d</sup>	Es	2009	FV	Santarém	1.00	1,8-Cineole 48, $\alpha$ -pinene 40
<i>Eucalyptus smithii</i> R.T. Baker <sup>d</sup>	Esm	2009	FV	Santarém	2.80	1,8-Cineole 83
<i>Eucalyptus urophylla</i> S. T. Blake <sup>d</sup>	Eu	2009	FV	Santarém	0.86	$\alpha$ -Phellandrene 45, 1,8-cineole 23
<i>Eucalyptus viminalis</i> Labill. <sup>d</sup>	Ev	2009	FV	Santarém	1.10	1,8-Cineole 46, $\alpha$ -pinene 13, $\gamma$ -terpinene 12
<i>Myrtus communis</i> L. <sup>b</sup>	Mco	2008	FF	Algarve	0.30	1,8-Cineole 37, $\alpha$ -pinene 24, limonene 13
<i>Syzygium aromaticum</i> (L.) Merrill and Perry	Sa	2010	Dfb	Herbal shop	9.00	Eugenol 92
<b>Pittosporaceae</b>						
<i>Pittosporum undulatum</i> Vent. 1 <sup>a</sup>	Pu1	2008	Ffruit	Graciosa, Azores	0.21	Sabinene 31, terpinen-4-ol 21, limonene 14, $\gamma$ -terpinene 10
<i>Pittosporum undulatum</i> Vent. 2 <sup>a,b</sup>	Pu2	2008	FL	Graciosa, Azores	0.08	Limonene 22, sabinene 18, terpinen-4-ol 15, $\gamma$ -terpinene 12
<b>Poaceae/Gramineae</b>						
<i>Cymbopogon citratus</i> (DC) Stapf. 1 <sup>a</sup>	Cci1	2008	FL	Faro	0.80	Geranial 43, neral 29, $\beta$ -myrcene 25
<i>Cymbopogon citratus</i> (DC) Stapf. 2	Cci2	2010	DL	Herbal shop	1.16	$\beta$ -Myrcene 38, geranial 23, neral 20, geraniol 14
<i>Cymbopogon citratus</i> (DC) Stapf. 3	Cci3	2010	DL	Herbal shop	3.04	Geranial 34, neral 22, $\beta$ -myrcene 20, geraniol 18
<b>Rutaceae</b>						
<i>Citrus limon</i> (L.) Burm. f. 1 <sup>b</sup>	CI1	2009	FF	Lisbon	0.22	$\beta$ -Pinene 34, limonene 32
<i>Citrus limon</i> (L.) Burm. f. 2 <sup>b</sup>	CI2	2009	Fex	Lisbon	0.32	Limonene 52, 1,8-cineole 17, $\beta$ -pinene 14
<i>Citrus limon</i> (L.) Burm. f. Var. Meyer 3	CI3	2009	Fex	Algarve	0.25	Limonene 45, 1,8-cineole 15, $\beta$ -pinene 14
<i>Citrus sinensis</i> (L.) Osbeck 1 <sup>b</sup>	Cs1	2009	Ffl	Lisbon	0.14	Sabinene 47, limonene 10
<i>Citrus sinensis</i> (L.) Osbeck 2 <sup>b</sup>	Cs2	2009	FV	Lisbon	0.26	Sabinene 64
<i>Citrus sinensis</i> (L.) Osbeck 3 <sup>b</sup>	Cs3	2009	Fex	Algarve	0.34	Limonene 81, $\beta$ -phellandrene 14
<i>Citrus sinensis</i> (L.) Osbeck var Valencia Late 4	Cs4	2009	Fex	Algarve	0.45	Limonene 78, $\beta$ -phellandrene 13
<i>Ruta graveolens</i> L. 1 <sup>a</sup>	Rg1	2009	FV	Évora	2.60	2-Undecanone 94
<i>Ruta graveolens</i> L. 2 <sup>a</sup>	Rg2	2009	DF	Herbal shop	0.90	2-Undecanone 93
<i>Ruta graveolens</i> L. 3	Rg3	2010	DV	Herbal shop	0.51	2-Undecanone 91
<b>Verbenaceae</b>						
<i>Aloysia citriodora</i> Gómez Ortega and Palau <sup>a,b</sup>	Ac	2009	DV	Herbal shop	0.19	Geranial 12, limonene 11, neral 10
<b>Zingiberaceae</b>						
<i>Zingiber officinale</i> Roscoe <sup>b</sup>	Zo	2008	Frhiz	Herbal shop	0.16	Geranial 29, $\beta$ -phellandrene 17, citronellol 14, camphene 14

DF – dry, flowering phase aerial parts; Dfb – dry flower buds; DL – dry leaves; DV – dry, vegetative phase aerial parts; Fex – fresh exocarp; FF – fresh, flowering phase aerial parts; Ffl – fresh flowers; Ffruit – fresh fruit; FL – fresh leaves; Frhiz – fresh rhizome; FV – fresh, vegetative phase aerial parts.

<sup>a</sup> EOs previously tested (Barbosa et al., 2010, 2012).

<sup>b</sup> Evaluated decoction waters.

<sup>c</sup> EO resulted from the combination of several EOs from the same chemotype collected in Azores from 2004 to 2009.

<sup>d</sup> EOs composition previously reported (Faria et al., 2011).

<sup>e</sup> Identification based on mass spectra only.

At the lowest concentration tested, 0.25  $\mu$ L/mL, *R. graveolens*, *S. montana*, and *T. capitata* EOs revealed to be the most active. The lethal doses (LC<sub>100</sub>) of these EOs were calculated, since the PWN has a high reproductive capability and can proliferate even from a very small population. LC<sub>100</sub> ranged from 0.358 to 0.544  $\mu$ L/mL for *R. graveolens* EOs, 0.374  $\mu$ L/mL for *S. montana* 2 EO and 0.375  $\mu$ L/mL for *T. capitata* EO (Table 3).

Previous studies (Barbosa et al., 2010, 2012) have shown similar high nematocidal potential, i.e. low LC<sub>100</sub>, for *O. vulgare*, *S. montana*, *T. capitata*, *Th. caespitius* (all rich in carvacrol,  $\gamma$ -terpinene and *p*-cymene) and 2-undecanone-rich *R. graveolens* EOs.

*R. graveolens* EOs herewith studied showed very good *B. xylophilus* anti-nematodal activity. *Ruta chalepensis* EOs, also 2-undeca-

none rich, showed also high activity against the root knot nematodes *Meloidogyne incognita* and *M. javanica* (Ntalli et al., 2011a).

Other thymol and carvacrol-rich essential oils have also been reported to have high *B. xylophilus* anti-nematodal activity. Thymol-rich *Th. vulgaris* EOs showed a good activity in direct contact assays against PWN (Kong et al., 2007). These EOs showed to be composed of both PWN propagation stimulant- and nematocidal compounds. Kong et al. (2007) showed that geraniol, thymol, carvacrol and terpinen-4-ol possessed strong nematocidal properties and (–)-caryophyllene oxide, (+)-ledene, (+)-limonene, (–)-limonene, linalool oxide,  $\beta$ -myrcene, (–)- $\alpha$ -phellandrene, (+)- $\alpha$ -pinene and  $\gamma$ -terpinene were PWN propagation stimulant compounds.



**Table 2**Main components ( $\geq 10\%$ ) and mean corrected mortality at 2  $\mu\text{L/mL}$  (mean  $\pm$  s.e., in %) of the EOs fractions.

Plant species <sup>a</sup>	EOs hydrocarbon molecules fraction main components	Corrected mortality (%)	EOs oxygen-containing molecules fraction main components	Corrected mortality (%)
<i>Cymbopogon citratus</i> 3	$\beta$ -Myrcene 72	58 $\pm$ 6	Geranial 45, neral 36	100 $\pm$ 0
<i>Origanum vulgare</i> 2	$\gamma$ -Terpinene 36, <i>p</i> -cymene 11	14 $\pm$ 11	$\alpha$ -Terpineol 26, thymol 23, terpinen-4-ol 16, carvacrol 15, linalool 14	100 $\pm$ 0
<i>Satureja montana</i> 2	$\gamma$ -Terpinene 44, <i>p</i> -cymene 19	53 $\pm$ 13	Carvacrol 96	100 $\pm$ 0
<i>Thymbra capitata</i>	$\gamma$ -Terpinene 36, <i>p</i> -cymene 23	39 $\pm$ 38	Carvacrol 93	100 $\pm$ 0
<i>Thymus caespitius</i> 6	<i>p</i> -Cymene 29, $\gamma$ -terpinene 16, <i>trans</i> -dehydroagarofuran 12	36 $\pm$ 21	Carvacrol 66, carvacrol acetate 14	100 $\pm$ 0

<sup>a</sup> The EOs that were chosen for fractionation showed corrected mortalities  $\geq 96\%$  at 2  $\mu\text{L/mL}$ .

*S. montana* 2 and *T. capitata* EOs, evaluated in the present study, are good candidates for EO fractionation since they contain several of the above cited compounds and, in addition, show high relative amounts of carvacrol and  $\gamma$ -terpinene.

To our knowledge this is the first report on several species EOs against the PWN. *Calamintha nepeta*, *Inula viscosa* and *O. majorana* EOs showed weak-, whereas *Genista tridentata* EO revealed moderate- and *O. vulgare* subsp. *virens* and *Mentha arvensis* EOs showed strong nematotoxic activities. From the *Eucalyptus* genus, eleven species and respective EOs were herewith tested for the first time against *B. xylophilus*, namely *E. bosistoana*, *E. botryoides*, *E. camaldulensis*, *E. cinerea*, *E. cordieri*, *E. ficifolia*, *E. pauciflora*, *E. polyanthemmos*, *E. saligna*, *E. urophylla* and *E. viminalis*.

*Eucalyptus citriodora* EO showed weak activity against the PWN in a previous study (Park et al., 2005). Nevertheless, the fact that Park et al. (2005) do not detail this EO composition does not allow a direct comparison between studies.

### 2.2.2. Essential oils and fractions containing hydrocarbons or oxygen-containing molecules

Given each specific EO composition, the PWN mortality and  $\text{LC}_{100}$  results, some essential oils were further chosen for fractionation, to evaluate the separate contribution of the EO fractions containing hydrocarbons or oxygen-containing molecules against the PWN.

Despite the high PWN mortality and  $\text{LC}_{100}$  results, *R. graveolens* EOs were not fractionated due to already high 2-undecanone content (Table 1). Thus, only *C. citratus* 3, *O. vulgare* 2, *S. montana* 2, *T. capitata* and *Th. caespitius* 6 EOs were fractionated (Table 2). These EO fractions with oxygen-containing molecules showed 100% corrected mortality at 2  $\mu\text{L/mL}$  (Fig. 1, Table 2). At the same concentration, the corresponding hydrocarbon fractions showed corrected mortalities  $\leq 58\%$  (Fig. 1, Table 2).

Cluster analysis (Fig. 1) showed that all fractions with oxygen-containing molecules grouped close to their corresponding EOs ( $S_{\text{corr}} \geq 0.8$ ), because of the dominance of oxygen-containing compounds, both in the fraction and in the original EO. On the contrary, with the exception of *C. citratus* 3, the hydrocarbon fractions clustered together ( $S_{\text{corr}} \geq 0.7$ ), showing similar chemical compositions (Fig. 1).

Given the results above, only the fractions with oxygen-containing molecules  $\text{LC}_{100}$  values were determined (Table 3). *S. montana* 2 and *T. capitata* fractions with oxygen-containing molecules revealed quite similar  $\text{LC}_{100}$  values to those of their corresponding EOs. On the other hand, *C. citratus* 3 oxygen-containing molecules fraction showed higher lethal dose value, than that of the related EO, even though the oxygen-containing compounds were present in higher proportions in the fraction. This suggests that in addition to the oxygen-containing compounds, the hydrocarbon fraction also plays an important role in the overall PWN toxicity of these

EOs, probably by additive and/or synergic interactions between EO fractions or compounds.

Unlike previous fractions, *O. vulgare* 2 and *Th. caespitius* 6 fractions with oxygen-containing molecules showed lower  $\text{LC}_{100}$  values comparatively to the related EOs. The diverse results obtained suggest that *B. xylophilus* toxicity may be EO specific, and no general conclusions should be drawn solely from the EOs main components.

Other studies have addressed the nematotoxic potential of separate EO components. In direct contact assays against PWN, Choi et al. (2007b) evaluated the activity of different monoterpenes, using synthetic chemicals. Out of the 26 monoterpenes tested, carvacrol, thymol, geraniol, nerol, (–)-menthol, citronellol, citronellal and citral (mixture of geranial and neral) showed the highest activities against PWN. By relating the anti-nematode activity to the chemical functional group, Choi et al. (2007b) showed that monoterpene hydrocarbons and ketones had weak or no activity. Other monoterpenes revealed a hierarchy of functional groups, phenols, aldehydes and primary alcohols being the most active followed by secondary and tertiary alcohols. Ntalli et al. (2011b) analyzed the synergistic and antagonistic interactions between components from EOs active against *Meloidogyne incognita*, showing that combinations of nematocidal EO components, such as carvacrol/thymol or carvacrol/geraniol, had a synergistic activity on *M. incognita* J2 juvenile paralysis.

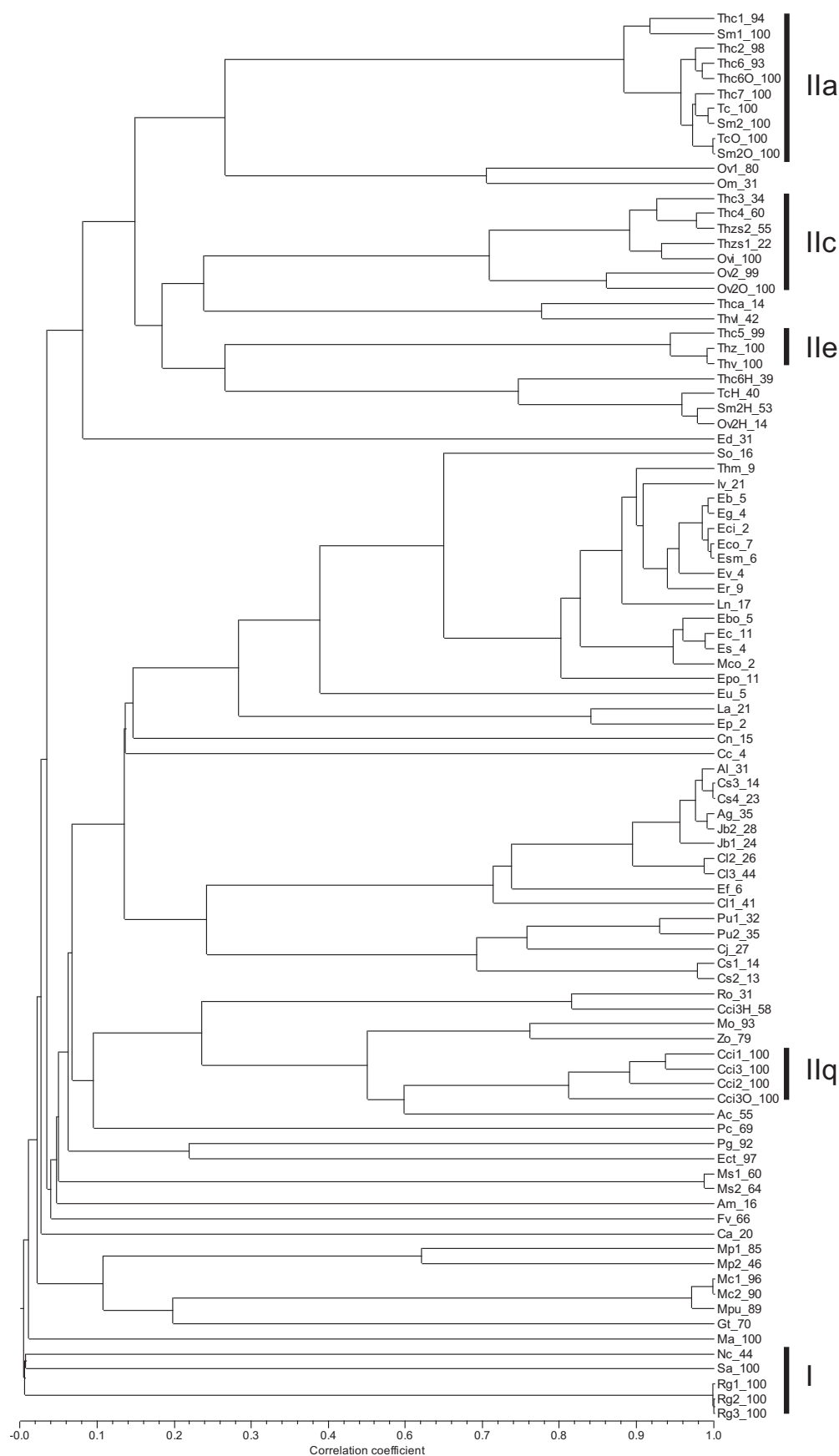
Although in the present study the nematotoxic activity of separate components was not assessed, the evaluation of the isolated fractions containing hydrocarbons or oxygen-containing molecules against PWN showed that the activity of an EO reflects the contribution of its different components, in distinct ways. Synergistic or antagonistic interactions between the EO components will influence the EO overall toxicity against PWN.

### 2.3. Decoction waters

Nine decoction waters (remaining hydrodistillation waters), out of the 45 evaluated (Table 1), showed strong nematotoxic activity, at 12.5  $\mu\text{L/mL}$ . Only *Angelica lignescens*, *Citrus sinensis* (flowers and fresh vegetative aerial parts), *F. vulgare*, *Laurus nobilis*, *Melissa officinalis*, *M. spicata*, *Salvia officinalis* and *Zingiber officinale* decoction waters revealed 100% PWN mortality.

Interestingly, with the exception of *M. officinalis* EO, the EOs isolated from the remaining samples with successful decoction waters, showed corrected mortalities  $\leq 80\%$ , at 2  $\mu\text{L/mL}$ .

Further studies are required to assess the active chemical constituents of these decoction waters. Decoction waters generally retain water-soluble, heat-stable, nonvolatile phytochemicals, such as high-weight terpenes and phenols and alkaloids (Gonçalves et al., 2009; Tiwari et al., 2011), which have been shown to possess strong nematocidal activities (Zhao, 1999; Chitwood, 2002).



**Fig. 1.** Dendrogram obtained by cluster analysis of the percentage composition of essential oils from the 84 samples and 10 fractions evaluated, based on correlation and using unweighted pair-group method with arithmetic average (UPGMA). For each EO sample abbreviation, see Table 1. EOs fractions abbreviations begin with the sample code followed by uppercase H for fractions containing hydrocarbon molecules or uppercase O for oxygen-containing molecules. Values after underscore are the mean corrected mortality percentages obtained with each EO or fraction at 2  $\mu\text{L/mL}$ .

**Table 3**  
Lethal concentrations (LC<sub>100</sub>, µL/mL) of EOs and related fractions with oxygen-containing molecules against the PWN. EC<sub>50</sub> and slope values are given for comparison purposes.

EOs/fractions with oxygen-containing molecules	Code	LC <sub>100</sub>	EC <sub>50</sub>	Slope (b <sup>a</sup> )
<i>Cymbopogon citratus</i> 3	Cci3	1.059	0.456	10.930
<i>Cymbopogon citratus</i> 3 O	Cci3O	1.801	0.454	6.685
<i>Origanum vulgare</i> 2	Ov2	2.120	0.754	8.909
<i>Origanum vulgare</i> 2 O	Ov2O	1.606	0.811	13.480
<i>Ruta graveolens</i> 1	Rg1	0.359	0.232	21.080
<i>Ruta graveolens</i> 2	Rg2	0.544	0.184	8.490
<i>Ruta graveolens</i> 3	Rg3	0.358	0.230	20.780
<i>Satureja montana</i> 2	Sm2	0.374	0.261	25.630
<i>Satureja montana</i> 2 O	Sm2O	0.374	0.262	25.850
<i>Thymbra capitata</i>	Tc	0.375	0.265	26.660
<i>Thymbra capitata</i> O	TcO	0.387	0.275	26.950
<i>Thymus caespititius</i> 6	Thc6	1.464	0.972	22.470
<i>Thymus caespititius</i> 6 O	Thc6O	0.721	0.471	21.610

O – EO oxygen-containing molecules fraction.

<sup>a</sup>  $b$  in  $y = C + (D - C) / (1 + \exp\{b[\log(x) - \log(EC_{50})]\})$ .

### 3. Conclusion

Essential oils and decoction waters, isolated from 84 samples, were evaluated through direct contact assays against PWN. Twenty highly nematocidal EOs were obtained from the initial screening. Of these *Ruta graveolens*, *Satureja montana* and *Thymbra capitata* EOs were the most active. Nematotoxic activity from fractions containing hydrocarbons or oxygen-containing molecules from the most successful EOs were further assessed, separately. For some EOs, fractionation may prove to be a good way to improve nematotoxic activity.

The use of EOs for pest management against the PWN must take into consideration the unique characteristics of each essential oil, as not every EO component contributes similarly against the PWN. The results obtained suggest that the EO fractions with oxygen-containing molecules, and some components, namely 2-undecanone, carvacrol, thymol, *p*-cymene and/or  $\gamma$ -terpinene may be responsible for EO nematotoxic activity. Nevertheless, despite the overall low activity of the EOs hydrocarbon fraction, this type of components also seems to contribute, in several cases, to the EOs PWN total nematotoxic activity, probably by additive and/or synergic interactions between EO fractions or compounds.

PWN nematotoxic phytochemicals will be further evaluated to determine highly active formulations of EOs, EO fractions, EO individual components, and/or decoction waters aimed at an integrated action against Pine Wilt Disease.

PWD control strategies can be accomplished in several ways and many times the strategies of management should be combined. Keeping in mind the expensive and labour-intensive work, as well as the possibility of chemical injury, the most effective compounds should be tested by trunk injection, in a preliminary assessment, both in healthy trees, as a preventive measure, as well as in already affected trees to evaluate the host and the pathogen response under field conditions.

### 4. Material and methods

#### 4.1. Plant material

Collective and/or individual samples, from cultivated and wild-growing medicinal and aromatic plants, were collected from mainland Portugal and at the Azores archipelago (Portugal) (Table 1). Dried aerial parts from commercially available products sold in local herbal shops were also analyzed. Thirteen families were sampled, from a total of 84 samples.

For all plants collected from the wild state a voucher specimen of each plant species was deposited in the Herbarium of the Botan-

ical Garden of Lisbon University, Lisbon, Portugal. For commercially obtained plant material, a reference sample from each plant is retained in our laboratory and is available on request.

#### 4.2. Essential oil extraction

Essential oils (EOs) were isolated by hydrodistillation for 3 h using a Clevenger type apparatus according to the European Pharmacopoeia (Council of Europe, 2010). Hydrodistillation was run at a distillation rate of 3 mL/min. EOs were stored in the dark at –20 °C, until analysis.

#### 4.3. Essential oil fractionation

Fractions containing hydrocarbons or oxygen-containing molecules were separated from each EO sample on a silica gel column [22 g of Silica gel 60 (Merck 9385) on a 85 mm internal diameter, 380 mm length column] by elution with 20 mL of distilled *n*-pentane (Riedel-de Haën, Sigma-Aldrich, Germany) followed by 20 mL diethyl ether (Panreac Química S.A.U., Barcelona, Spain), per mL of essential oil. A total of 5 mL of EO was fractionated. The hydrocarbon fraction was obtained after distilled *n*-pentane elution, and diethyl ether eluted the EO oxygen-containing components.

Both fractions were concentrated, separately, at room temperature under reduced pressure on a rotary evaporator (Yamato, Hitec RE-51), collected in a vial, and concentrated to a minimum volume, again at room temperature, under nitrogen flux. Fractions were then stored in the dark at –20 °C until analysis.

#### 4.4. Essential oil and fractions composition analysis

Essential oils and the corresponding fractions containing hydrocarbons or oxygen-containing molecules were analyzed by gas chromatography (GC), for component quantification, and gas chromatography coupled to mass spectrometry (GC–MS) for component identification, as detailed in Barbosa et al. (2010).

The percentage composition of the isolated EOs was used to determine the relationship between the different samples by cluster analysis using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc software, version 2.2, Exeter Software, Setauket, New York) (Rohlf, 2000). For cluster analysis, correlation coefficient was selected as a measure of similarity among all accessions, and the Unweighted Pair Group Method with Arithmetical Averages (UPGMA) was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro (2000) in

very high (0.9–1), high (0.7–0.89), moderate (0.4–0.69), low (0.2–0.39) and very low (<0.2).

#### 4.5. Isolation of decoction waters

After hydrodistillation, each of the 45 decoction waters (remaining hydrodistillation water) was separated from the plant material through coarse sieving using filter paper. The decoction waters were separately concentrated to a minimum volume, at 60 °C under reduced pressure, in a rotary evaporator. The concentrated decoction water (CDW), was stored in the dark at –20 °C until use.

#### 4.6. Nematode collection and rearing

Pinewood nematodes (PWNs) were obtained according to Barbosa et al. (2010). Axenic cultures of *Botrytis cinerea* (de Bary) Whetzel were grown for 7 days, at  $25 \pm 1$  °C, on steam-sterilized hydrated commercial barley grains (*Hordeum vulgare* L.). An aliquot of 100 µL, containing 100–200 mixed-stage PWNs, in ultrapure water, was then added to these cultures.

After 7–10 days, in darkness, at  $25 \pm 1$  °C, the PWN population, grown by consuming the fungus, was isolated by a modified Baermann funnel technique (Viglierchio and Schmitt, 1983). Live nematodes, which naturally descended to the bottom of the apparatus, were collected after 24 h into a 20 µm mesh sieve and rinsed thoroughly with ultrapure water. Nematode solutions were used for further inoculations or stored at 4 °C. PWN mortality assessment was performed using an inverted microscope [Diaphot, Nikon, Japan (40×)].

#### 4.7. Direct contact bioassays

Essential oils, fractions containing hydrocarbons or oxygen-containing molecules and concentrated decoction waters (CDW) were assayed in newly extracted nematode suspensions as detailed in Barbosa et al. (2010). EOs and fractions stock solutions were prepared in methanol (Panreac Química S.A.U., Barcelona, Spain), at 40 µL/mL. A previous study has shown that the use of water-miscible solvents such as acetone (Barbosa et al., 2012) are more advantageous than the widely used detergents, such as Triton X-100, when performing direct contact assays employing EOs. Methanol was presently chosen due to its high polarity and high solvent capacity.

To obtain a final concentration of 2 µL/mL, 5 µL of these solutions were added to 95 µL of nematode suspensions with 50–100 mixed stage nematodes. Stock solutions for 1, 0.5 and 0.25 µL/mL were obtained by serial dilutions with a dilution factor of two. The EOs which showed corrected mortalities below 96% were not further assayed at lower concentrations. Control trials were performed with methanol 5% (v/v, methanol/nematode suspension). In order to check for methanol induced mortality ultrapure water was used as corresponding control.

Stock solutions for the CDW were obtained by centrifuging a 1:1 mixture of CDW in ultrapure water (2500 G), to remove high weight debris. The supernatant was assayed at the final concentration, in the test suspension, of 25 µL of CDW per mL of nematode suspension. CDWs were assessed for the nematode's complete mortality only; if live nematodes were detected CDWs were not further assayed at lower concentrations. CDWs which showed complete mortality at one given concentration were tested at a lower one, 12.5 and 6.3 µL of CDW per mL of nematode suspension.

All bioassays were performed in flat bottom 96-well microtiter plates (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), covered with plastic film, to diminish volatilization, and aluminum foil to establish total darkness. The plates were maintained at  $25 \pm 1$  °C

in an orbital shaker at 90 r.p.m., for 24 h. Dead and live nematodes were counted under an inverted microscope (40×). Nematodes were considered dead if they did not move even when physically stimulated. A minimum of 10 assays were performed for each sample, in, at least, two separate trials.

#### 4.8. Lethal concentration (LC<sub>100</sub>) determination

The determination of the lowest concentration at which 100% death is observed (LC<sub>100</sub>), was based on mean corrected mortality values. Mean corrected mortality calculated in each trial, was obtained by comparing the percentage mortality due to essential oil treatment to the percentage mortality in the methanol control, using the Schneider-Orelli formula (Puntener, 1981):

$$\text{Corrected mortality \%} = \left[ \frac{(\text{Mortality \% in treatment} - \text{mortality \% in control})}{(100 - \text{mortality \% in control})} \right] \times 100$$

The application of this formula normalizes the mortality values, allowing the comparison of different runs with slightly different mortalities.

Nematotoxic activity was evaluated according to Kong et al. (2006) by classifying mortality as strong (>80%), moderate (80–61%), weak (60–40%) and low or inactive (<40%).

To estimate the half maximal effective concentration (EC<sub>50</sub>), mean corrected mortality values were subjected to non-linear regression analysis using a dose–response log–logistic equation proposed by Seefeldt et al. (1995):

$$y = C + (D - C) / 1 + \exp\{b[\log(x) - \log(\text{EC}_{50})]\}$$

which relates the average response  $y$  to dose  $x$ , and where  $C$  and  $D$  are, respectively, the lower- and the upper limit of the sigmoidal dose–response curve,  $b$  is the slope and  $\text{EC}_{50}$  is the EO or fraction concentration which induces a response halfway between the lower- and the upper limit. This analysis was performed using GraphPad Prism® version 5.00 for Windows, San Diego California USA (www.graphpad.com), setting  $C$  to 0% and  $D$  to 100% with variable slope ( $b$ ). LC<sub>100</sub> values were calculated using the GraphPad 5.0 software QuickCalcs (<http://www.graphpad.com/quickcalcs/Ecanything1.cfm>).

#### Acknowledgments

The authors would like to thank Prof. José Barroso, Prof. Helena Trindade, M.Sc.'s Marta D. Mendes, Sofia Lima, Natacha Moura and Marta Taveira (Faculdade de Ciências da Universidade de Lisboa, CBV, IBB), Prof. Graça Miguel (Faculdade de Ciências e Tecnologia, Universidade do Algarve, CBV, IBB), Dr. Leandra Rodrigues (Instituto Superior de Agronomia, Universidade Técnica de Lisboa), Prof. Kiril Bahcevandziev (Escola Superior Agrária de Coimbra), Cacial - Cooperativa Agrícola de Citricultores do Algarve, C.R.L. and Eng. João Sanches (Mata Experimental do Escaroupim, Instituto da Conservação da Natureza e das Florestas), for providing some of the plant material and/or for the technical support. Jorge Faria is grateful to the Fundação para a Ciência e a Tecnologia (FCT) for the Ph.D. grant SFRH/BD/43738/2008. This study was partially funded by FCT, under Pest-OE/EQB/LA0023/2011 and research contract PTDC/AGR-CFL/117026/2010.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phytochem.2013.06.005>.



## References

- Abelleira, A., Picoaga, A., Mansilla, J.P., Aguin, O., 2011. Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in northwestern Spain. *Plant Dis.* 95, 776.
- Back, M.A., Haydock, P.P.J., Jenkinson, P., 2002. Disease complexes involving plant parasitic nematodes and soilborne pathogens. *Plant Pathol.* 51, 683–697.
- Barbosa, P., Lima, A.S., Vieira, P., Dias, L.S., Tinoco, M.T., Barroso, J.G., Pedro, L.G., Figueiredo, A.C., Mota, M., 2010. Nematicidal activity of essential oils and volatiles derived from Portuguese aromatic flora against the pinewood nematode, *Bursaphelenchus xylophilus*. *J. Nematol.* 42, 8–16.
- Barbosa, P., Faria, J.M.S., Mendes, M.D., Dias, L.S., Tinoco, M.T., Barroso, J.G., Pedro, L.G., Figueiredo, A.C., Mota, M., 2012. Bioassays against pinewood nematode: assessment of a suitable dilution agent and screening for bioactive essential oils. *Molecules* 17, 12312–12329.
- Chitwood, D.J., 2002. Phytochemical based strategies for nematode control. *Annu. Rev. Phytopathol.* 40, 221–249.
- Chitwood, D.J., 2003. Nematicides. In: Plimmer, J.R. (Ed.), *Encyclopedia of Agrochemicals*, vol. 3. John Wiley Sons, New York, pp. 1104–1115.
- Choi, I.H., Park, J.Y., Shin, S.C., Kim, J., Park, I.K., 2007a. Nematicidal activity of medicinal plant essential oils against the pinewood nematode (*Bursaphelenchus xylophilus*). *Appl. Entomol. Zool.* 42, 397–401.
- Choi, I.H., Kim, J., Shin, S.C., Park, I.K., 2007b. Nematicidal activity of monoterpenoids against the pine wood nematode (*Bursaphelenchus xylophilus*). *Russ. J. Nematol.* 15, 35–40.
- Council of Europe, 2010. European directorate for the quality of medicines. In: *European Pharmacopoeia*, seventh ed., Strasbourg, France, p. 241.
- EPPO. (2012) Data sheets on quarantine pests: *Bursaphelenchus xylophilus*. <[http://www.eppo.int/QUARANTINE/nematodes/Bursaphelenchus\\_xylophilus/BURSXY\\_ds.pdf](http://www.eppo.int/QUARANTINE/nematodes/Bursaphelenchus_xylophilus/BURSXY_ds.pdf)> Accessed 15 January 2013>.
- Faria, J.M.S., Sanches, J., Lima, A.S., Mendes, M.D., Leiria, R., Gerales, D.A., Figueiredo, A.C., Trindade, H., Pedro, L.G., Barroso, J.G., 2011. *Eucalyptus* from Mata Experimental do Escarpim (Portugal): evaluation of the essential oil composition from sixteen species. *Acta Hort.* 925, 61–66.
- Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Scheffer, J.J.C., 2008. Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour Fragrance J.* 23, 213–226.
- Fonseca, L., Cardoso, J.M.S., Lopes, A., Pestana, M., Abreu, F., Nunes, N., Mota, M., Abrantes, I., 2012. The pinewood nematode, *Bursaphelenchus xylophilus*, in Madeira Island. *Helminthologia* 49, 96–103.
- GraphPad Prism® version 5.00 for Windows. GraphPad Software, San Diego, California, USA. Available at: <<http://www.graphpad.com>> (accessed 18.10.12).
- GraphPad Software, Inc. Available at: <<http://www.graphpad.com/quickcalcs/Ecanything1.cfm>> (accessed 18.10.12).
- Gonçalves, C., Dinis, T., Baptista, M.T., 2009. Antioxidant properties of proanthocyanidins of *Uncaria tomentosa* bark decoction: a mechanism for anti-inflammatory activity. *Phytochemistry* 66, 89–98.
- Kim, J., Seo, S.M., Lee, S.G., Shin, S.C., Park, I.K., 2008. Nematicidal activity of plant essential oils and components from coriander (*Coriandrum sativum*), oriental sweet gum (*Liquidambar orientalis*), and valerian (*Valeriana wallichii*) essential oils against pine wood nematode (*Bursaphelenchus xylophilus*). *J. Agric. Food Chem.* 56, 7316–7320.
- Kong, J.O., Lee, S.M., Moon, Y.S., Lee, S.G., Ahn, Y.J., 2006. Nematicidal activity of plant essential oils against *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). *J. Asia-Pac. Entomol.* 9, 173–178.
- Kong, J.O., Park, I.K., Choi, K.S., Shin, S.C., Ahn, Y.J., 2007. Nematicidal and propagation activities of thyme red and white oil compounds toward *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae). *J. Nematol.* 39, 237–242.
- Lee, S.M., Chung, Y.J., Moon, Y.S., Lee, S.G., Lee, D.W., Choo, H.Y., Lee, C.K., 2003. Insecticidal activity and fumigation conditions of several insecticides against Japanese pine sawyer (*Monochamus alternatus*) larvae. *J. Korean Forensic Soc.* 92, 191–198.
- Mota, M., Braasch, H., Bravo, M.A., Penas, A.C., Burgermeister, W., Metge, K., Sousa, E., 1999. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* 1, 727–734.
- Mota, M., Vieira, P., 2008. Pine Wilt Disease: A Worldwide Threat to Forest Ecosystems. Springer, Heidelberg, Germany.
- Ntalli, N.G., Manconi, F., Leonti, M., Maxia, A., Caboni, P., 2011a. Aliphatic ketones from *Ruta chalepensis* (Rutaceae) induce paralysis on root knot nematodes. *J. Agric. Food Chem.* 59, 7098–7103.
- Ntalli, N.G., Ferrari, F., Giannakou, I., Menkissoglu-Spiroudi, U., 2011b. Synergistic and antagonistic interactions of terpenes against *Meloidogyne incognita* and the nematicidal activity of essential oils from seven plants indigenous to Greece. *Pest. Manag. Sci.* 67, 341–351.
- Park, I.K., Park, J.Y., Kim, K.H., Choi, K.S., Choi, I.H., Kim, C.S., Shin, S.C., 2005. Nematicidal activity of plant essential oils and components from garlic (*Allium sativum*) and cinnamon (*Cinnamomum verum*) oils against the pine wood nematode (*Bursaphelenchus xylophilus*). *Nematology* 7, 767–774.
- Park, I.K., Kim, J., Lee, S.G., Shin, S.C., 2007. Nematicidal activity of plant essential oils and components from Ajowan (*Trachyspermum ammi*), Allspice (*Pimenta dioica*) and Litsea (*Litsea cubeba*) essential oils against pine wood nematode (*Bursaphelenchus xylophilus*). *J. Nematol.* 39, 275–279.
- Pestana, M.H., Gageiro, J.N., 2000. *Análise de dados para ciências sociais. A complementaridade do SPSS. Edições Sílabo, Lisboa.*
- Puntener, W., 1981. *Manual for Field Trials in Plant Protection*, 2nd edition. Ciba-Geigy Limited, Basle, Switzerland, p. 205.
- Rohlf, J.F., 2000. NTSYS-pc, Numerical Taxonomy and Multivariate Analysis System, version 2.1, user guide. Applied Biostatistics, New York.
- Seefeldt, S.S., Jensen, J.E., Fuerst, E.P., 1995. Log-logistic analysis of herbicide dose response relationships. *Weed Technol.* 9, 218–227.
- Takai, K., Suzuki, T., Kawazu, K., 2003. Development and preventative effect against pine wilt disease of a novel liquid formulation of emamectin benzoate. *Pest. Manag. Sci.* 59, 365–370.
- Tiwari, P., Kumar, B., Kaur, M., Kaur, H., 2011. Phytochemical screening and extraction: a review. *Int. Pharm. Sci.* 1, 98–106.
- Vicente, C.S.L., Nascimento, F., Espada, M., Mota, M., Oliveira, S., 2011. Bacteria associated with the pinewood nematode *Bursaphelenchus xylophilus* collected in Portugal. *Antonie Van Leeuwenhoek J. Microb.* 100, 477–481.
- Viglierchio, D.R., Schmitt, R.V., 1983. On the methodology of nematode extraction from field samples: Baermann funnel modifications. *J. Nematol.* 15, 438–444.
- Zhao, B.G., 1999. Nematicidal activity of quinolizidine alkaloids and the functional groups pairs in their molecular structure. *J. Chem. Ecol.* 25, 2205–2214.
- Zhao, B.G., 2008. Pine Wilt Disease in China. In: Zhao, B.G., Futai, K., Sutherland, J.R., Takeuchi, Y. (Eds.), *Pine Wilt Disease*. Springer, Tokyo, Japan, pp. 18–25.